REMARKS

Claims 1-5, 31, and 33-35 are pending and under examination.

Rejection Under 35 U.S.C. § 101

The rejection of claims 1-5, 31 and 33-35 under 35 U.S.C. § 101 as allegedly lacking utility is respectfully traversed. Applicants respectfully maintain, for the reasons of record, that the claimed nucleic acid molecules have a specific, substantial and credible utility.

In the Office Action on page 3, it is acknowledged that CARD proteins such as CLAN-A (SEQ ID NO:97; see Table 1, page 8) interact with CARD proteins in ways that "likely influence apoptosis, cytokine processing, or NFkB activity," referring to paragraph 0043 of the specification, and have a credible utility. However, the Office Action asserts on page 3 that there is no evidence of any substantial utility. The Office Action on pages 3-4 further asserts that "the specification indicates that the Clan molecules can have opposing functions, so that some Clan molecules may trigger pro-caspase-1 activation while others may inhibit this activation. Further, even if the phenotype is pro-caspase-1 activation, this phenotype does not meet the requirements for a 'substantial' utility since the specification provides no information on how to use such a phenotype." With regard to this assertion, it appears to be based on the teaching in the specification on page 16.

> As also described in the Example, because of their interactions with diverse other CARD proteins, the isoforms of CLAN (A, B, C and D) likely influence apoptosis, cytokine processing, or NF-kB activity. Interactions of CLAN with pro-caspase-1 likely indicates a role for CLAN as a IL-1β regulator. In this regard, different isoforms of CLAN likely have opposing effects on pro-caspase-1 activation. The longest isoform, CLAN-A, for example, can trigger pro-caspase-1 activation by the "induced proximity" mechanism as a result of oligomerization mediated by its NB-ARC (NACHT) domain. In contrast, the shorter isoforms of CLAN lacking this self-oligomerization can operate as competitive antagonists of pro-caspase-1 activation, analogous to ICEBERG, a CARD-containing protein that competes with CARDIAK (RIP2/RICK) for binding to pro-caspase-1. Interactions of CLAN with NAC also suggest this protein can have an influence on apoptosis mediated by Apaf-1, in as much as NAC binds Apaf-1 and enhances its ability to activate caspase-9 in response to cytochrome c. Finally, CLAN associations with NF-kB regulators such as Bcl-10 and Nod2 strongly suggest that at least some of

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> the CLAN isoforms can influence the activity of this transcription factor. [specification page 16, line 17, to page 17, line 8]

Thus, the assertion in the Office Action that "Clan molecules can have opposing functions" appears to relate to the teaching in the specification that isoforms of CLAN such as CLAN-A can trigger pro-caspase-1 activation whereas shorter isoforms of CLAN lacking the self-oligomerization NB-ARC (NACHT) domain can operate as competitive antagonists of pro-caspase-1. However, the differing activities of the isoforms of CLAN are irrelevant to the claimed nucleic acids, which are directed to the CLAN-A isoform referenced as SEQ ID NO:97. Thus, there is no ambiguity as to the asserted activity of the claimed CLAN-A encoding nucleic acid molecules.

Referring to the assertion in the Office Action on page 4 that even the activation of pro-caspase-1 would not be a phenotype meeting the requirements for a "substantial" utility, Applicants respectfully disagree with the assertion that the specification provides no information on how to use a pro-caspase-1 activation phenotype. To the contrary, the specification teaches that interactions of CLAN (i.e. CLAN-A) with pro-caspase-1 likely indicates a role for CLAN as a IL-1β regulator (page 16, lines 21-22). The specification further teaches that IL-1β is a pro-inflammatory cytokine (page 11, lines 1-2; page 16, lines 8-10). Thus, the specification clearly teaches that CLAN-A can activate pro-caspase-1 and play a role in regulating the pro-inflammatory cytokine IL-1β.

In corroboration of the specific, substantial and credible utility of the claimed nucleic acid molecules encoding CLAN-A (SEQ ID NO:97), submitted herewith as Exhibit 1 is Damiano et al., Genomics 75:77-83 (2001)(hereinafter Damiano et al., 2001), which has been previously discussed on the record and is addressed with respect to the assertions in the Office Action about this reference in more detail below. Damiano et al., 2001, substantially discloses aspects of the present invention (see Example, page 114, line 19, to page 124, line 4). In particular, Damiano et al., 2001, describes cloning of CLAN and identification of four isoforms, including CLAN-A. The CARD domain of CLAN was shown to bind to pro-caspase-1 (page 80, last paragraph). Damiano et al., 2001, also discloses that interactions of CLAN with pro-caspase-1 can be indicative of a role for CLAN as an interleukin-1β regulator (page 82, first paragraph).

As further corroboration of the specific, substantial and credible utility of the claimed nucleic acid molecules encoding CLAN-A (SEQ ID NO:97), submitted herewith as Exhibit 2 is Damiano et al., J. Immunol. 173:6338-6345 (2004)(hereinafter Damiano et al., 2004). Damiano et al., 2004, describes multiple roles of CLAN in the mammalian innate immune response. Damiano et al., 2004, discloses that CLAN modulates endogenous caspase-1 activation and subsequent IL-1β secretion from human macrophages after exposure to lipopolysaccharide (LPS), peptidoglycan, and pathogenic bacteria (see abstract; page 6339, last paragraph of Introduction; page 6340, right column, to page 6341, left column; page 6342, right column). Thus, Damiano et al., 2004, corroborates the teaching in the specification that CLAN-A activates caspase-1 and is an IL-1β regulator.

Applicants respectfully disagree with the assertion in the Office Action on page 4 that Damiano et al., 2001, supports a conclusion that there is no "real world" use for SEQ ID NO:97 other than further investigation. The Office Action refers to the last sentence of the abstract, "[O]nce their physiologic functions are uncovered, CLAN proteins may prove to be valuable therapeutic targets" and asserts that "at a time later than that of the submission of this application" Applicant indicates that the physiologic function of CLAN proteins are unknown and "may" be valuable targets. Contrary to the assertion in the Office Action and as discussed above and in the previous response, Damiano et al., 2001, is in agreement with the teaching in the specification that CLAN-A interacts with and activates pro-caspase-1 and plays a role as an IL-1β regulator, a pro-inflammatory cytokine. This utility is further corroborated by Damiano et al., 2004.

Applicants respectfully disagree with the assertion in the Office Action on page 4 that the cited utilities of pro-caspase-1 activation or inhibition have less "real world" significance than the amount of utility found insufficient in Brenner v. Manson, 148 USPQ 689 (1966). First, Applicants point out that the utility of the claimed CLAN-A (SEQ ID NO:97) is activation of pro-caspase-1 and regulation of IL-1β, not "inhibition." Second, as discussed in the previous response, the present specification recites a specific, substantial and credible utility, activation of pro-caspase-1 and regulation of IL-1β, a pro-

inflammatory cytokine, in contrast to the facts in Brenner v. Manson, where no utility was asserted.

In the Office Action on page 5, the specification is asserted to discuss a wide variety of phenotypes that might be influenced by CLAN-A, SEQ ID NO:97, such as cytokine processing, NF-kB activity or apoptosis. However, Applicants respectfully disagree with the assertion in the Office Action on page 6 that the specification does not specifically teach any use for the sequence in association with these multiple "generic possibilities." The Office Action further asserts that "[E]ven the claims are drawn to generic utilities as shown by nonelected claim 23, where the biological process includes elements ranging from apoptosis to inflammation, cell adhesion and, most generic of all, transcription." First with respect to this assertion, it is unclear as to what is intended by the term "generic utility." Upon a text search of MPEP § 2107, which relates to the Guidelines for Examination of Applications for Compliance with the Utility Requirement, the term "generic" was only found in relation to a claim, and no such term as "generic utility" was found. It is unclear what a "generic utility" would be. Nevertheless, Applicants respectfully submit that, contrary to the assertion in the Office Action, the specification does teach the use of the claimed CLAN-A sequence, SEQ ID NO:97. In particular, the specification teaches that the CARD of CLAN binds to pro-caspase-1 and CLAN-A can trigger pro-caspase-1 activation, that CLAN interacts with NAC and can have influence on apopotosis mediated by Apaf-1, and that CLAN associates with NFkB regulators such as Bcl-10 and Nod2 and can influence the activity of this transcription factor (page 16, line 17, to page 17, line 8; page 123, line 27, to page 124, line 4). Thus, the activities taught in the specification are based on experimentally determined binding activities. Accordingly, the so-called "generic utilities" of claim 23 are based on experimentally determined binding activities, including binding to pro-caspase-1, supporting "cytokine processing," "caspase-mediated proteolysis" and "inflammation" per the role of pro-caspase-1 in regulating processing of IL-1β; and binding to the transcription factor NF-kB, supporting "NF-kB induction," "cJun N-terminal kinase induction," and "transcription." The fact that a number of uses are asserted, for example, as recited in claim 23, including some considered "generic," is

irrelevant to the utility requirements. As indicated in MPEP § 2107(II)(B)(1)(ii), "[A]n applicant need only provide one credible assertion of specific and substantial utility for each claimed invention to satisfy the utility requirement" [emphasis added].

Moreover, Applicants respectfully disagree with the assertion in the Office Action on page 6 that "the current situation directly tracks Example 9 of the utility guidelines, where a nucleic acid of significantly unknown function was characterized as lacking utility." Turning to Example 9 of the utility guidelines, Example 9 relates to a specification disclosing 4332 nucleic acid sequences that are fragments of full length genes isolated from a human cDNA library. The specification discloses how to use each of the 4332 nucleic acid sequences as a probe to obtain the full length genes that correspond to the nucleic acid sequences, which can be used to recombinantly make corresponding proteins, which can be used to study the cellular mechanisms and activities in which the proteins are involved. No use is disclosed for any of the putative proteins other than the possibility of using them to identify and study the cellular mechanisms and activities in which the proteins are involved. Clearly the claimed nucleic acid molecule encoding CLAN-A, SEQ ID NO:97, does not fall within the fact pattern set forth in Example 9 of the utility guidelines. In contrast to the numerous nucleic acid fragments that are uncharacterized and asserted to function as probes to obtain full length sequences as in Example 9 of the utility guidelines, the claimed nucleic acid is directed to a full length cDNA, in this case the longest CLAN isoform, CLAN-A. Furthermore, the CLAN-A sequence has been characterized with respect to containing specific motifs, namely a CARD domain, an NB-ARC (NACHT) domain, an LRR domain and a SAM domain (Table 1, page 8; page 14, lines 8-10; page 118, line 1, to page 119, line 12). In addition to the activity of the CARD domain of CLAN binding to pro-caspase-1, the CARD of CLAN also interacts with the CARDs of Nod2 and NAC and with Bcl-10 (page 124, lines 1-4). Furthermore, the claimed nucleic acid molecules have a specific, substantial and credible utility, as discussed above, for example, interacting with pro-caspase-1 and playing a role as an IL-1β regulator (page 16, lines 21-22). Thus, contrary to the assertion in the Office Action, clearly the fact pattern of Example 9 in the utility

guidelines is not relevant to the claimed nucleic acid molecule having the specific, substantial and credible utility as taught in the specification.

For the reasons of record and the reasons discussed above, Applicants respectfully submit that the claimed nucleic acid molecules encoding SEQ ID NO:97 (CLAN-A) has a specific, substantial and credible utility. Accordingly, Applicants respectfully request that this rejection be withdrawn.

Rejection Under 35 U.S.C. § 112, First Paragraph

The rejection of claims 1-5, 31 and 33-35 under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement is respectfully traversed. Applicants respectfully maintain, for the reasons of record, that the specification provides sufficient description and guidance to enable the claimed nucleic acid molecules.

Applicants wish to clarify certain remarks in the Office Action. First, it is respectfully pointed out that the claims under examination are directed to nucleic acid molecules encoding SEQ ID NO:97 (CLAN-A), vectors containing the nucleic acid molecules, and recombinant cells containing the nucleic acid molecules, not a method of screening using SEQ ID NO:97 as asserted in the Office Action on page 6 under "Nature of Invention." Further, Applicants respectfully disagree with the assertion in the Office Action that the nature of the invention relates to nucleic acids of a particular sequence "with no other associated information." To the contrary and as discussed above, the claimed nucleic acid molecule encodes CLAN-A (SEQ ID NO:97), which has been characterized as containing a CARD domain, NB-ARC/NACHT domain, LRR domain and SAM domain (Table 1, page 8; page 14, lines 8-10; page 118, line 1, to page 119, line 12). Furthermore, the CARD domain of CLAN was shown to bind to pro-caspase-1 (page 123, lines 27-29). Thus, the claimed nucleic acid molecules and encoded polypeptide CLAN-A clearly have associated functional information.

Applicants respectfully disagree with the assertion on page 7 that the specification identifies no particular use for SEQ ID NO:97. To the contrary and as discussed above, the specification teaches that the CARD domain of CLAN binds to pro-caspase-1 and

that CLAN-A can trigger pro-caspase-1 activation and is an IL-1β regulator. Further as discussed above, the claimed nucleic acid molecules do have a specific, substantial, and credible utility, as taught in the specification and corroborated by Exhibits 1 and 2. With regard to the assertion in the Office Action on page 7 that the specification indicates that different isoforms of CLAN likely have opposing effects on pro-caspase-1 activation and therefore the function or use of the molecule based on the sequence is unpredictable, Applicants respectfully point out, as discussed above, that the issue of whether different forms of CLAN other than the claimed CLAN-A isoform have different activities is irrelevant because the claims are directed to a specific isoform, CLAN-A (SEQ ID NO:97), and no issue of unpredictability of the claimed nucleic acid molecule has been established.

In the Office Action on page 8, it is asserted that Applicants' own paper, referring to Damiano et al., 2001, supports a conclusion that there is no "real world" use other than further investigation for SEQ ID NO:97. The last sentence of the abstract is referred to as supporting that "even Applicant, at a time later than that of the submission of this application, indicates that the physiologic functions of CLAN proteins are unknown, and they 'may' be valuable targets." First with respect to the assertion that "at a later time" than the submission of the present application the physiologic functions of CLAN were unknown, it is pointed out that the present application was filed May 23, 2001, claiming priority to several provisional applications filed as early as May 24, 2000. Damiano et al., 2001, was published in July 2001. While July of 2001 is clearly "at a later time" than May, 24, 2001, the time of the filing of the present application was substantially similar to the publication of Damiano et al., 2001, and, as discussed above, Damiano et al., 2001, discloses subject matter of the present application. Further as discussed above, Damiano et al., 2001, is in agreement with the teaching in the specification that CLAN-A interacts with and activates pro-caspase-1 and plays a role as an IL-1β regulator, a pro-inflammatory cytokine. Whether or not Damiano et al., 2001, further speculates on additional physiologic functions of CLAN is irrelevant to the claimed nucleic acids having a use as an activator of pro-casepase-1 and regulator of IL-1β, as taught in the

specification and corroborated by Damiano et al., 2001. Such a use is further corroborated by Damiano et al., 2004 (Exhibit 2).

In the Office Action on page 8, the claimed sequence is asserted to be an "orphan gene," and Dujon, Trends Genet. 12:263-270 (1996), is referred to as describing that a significant proportion of yeast genes are orphans of unpredictable function. As discussed in the previous response, Applicants respectfully disagree with the assertion that SEQ ID NO:97 (CLAN-A) is an orphan gene. Dujon describes, under "The mystery of orphans" (page 266, left column), that the "most striking result from the chromosome III sequence was that approximately half of all protein-coding ORFs revealed by the sequence, had no clearcut sequence homologs in any organism, including yeast itself" [emphasis addeed]. Thus, an orphan gene is considered to be an open reading frame (ORF) with no homology to a known sequence. This is corroborated by Exhibit 3, a printout from the "EverythingBio" website (http://www.everythingbio.com) providing the definition of an orphan gene as "putative ORFs without any resemblance to previously determined protein coding sequences" [emphasis added]. Applicants maintain that CLAN-A, SEQ ID NO:97, cannot be considered an orphan gene as understood by those skilled in the art. To the contrary, the specification teaches that CLAN represents a new member of the CED-4 related protein family (page 14, lines 10-11). Further, CLAN was found to contain a CARD domain, LRR domain, NB-ARC (NACHT) domain and SAM domain (Table 1, page 8; page 118, line 1, to page 119, line 12). In addition, the CARD of CLAN was shown to bind to pro-caspase-1 (page 123, lines 27-29). Thus, the claimed nucleic acid encoding CLAN-A (SEQ ID NO:97) would not be considered an orphan gene by one skilled in the art. Accordingly, Applicants respectfully submit that the comments in the Office Action on Dujon and orphan genes is irrelevant to the claimed nucleic acid molecules encoding CLAN-A.

The Office Action on page 9 further refers to Rost et al., J. Mol. Biol. 318:595-608 (2002), as providing evidence that very similar proteins can perform very different functions. Rost et al. is noted for discussing that the assignment of enzymatic activity based on homology comparisons is difficult. However, Applicants note that Rost et al. is specifically referring to the difficulty of assigning an enzymatic function based on protein

homology. It is notable that, in reviewing the examples of "enzymes of similar sequence and different function" described in Table 1, though many have different specific enzymatic activities, many of the "different" activities have similar mechanisms, including many reactions involving ATP. As one example, Pair 9 of "similar sequences" having "different function" (Table 1, page 600) compares a tyrosine kinase with a tyrosine phosphatase, an example of a "different function" that is in fact the reverse reaction, therefore an example of a "different" enzymatic reaction that understandably has a similar sequence given the similarity of the reaction, albeit the reverse reaction, being carried out. Furthermore, the claimed CLAN-A contains CARD, NB-ARC (NACHT), LRR and SAM domains, which are binding domains, not domains asserted to have enzymatic activity. Therefore, the discussion in Rost et al. of the alleged unpredictability of enzyme activity based on sequence homology is not relevant to the claimed nucleic acid molecule encoding CLAN-A containing binding domains, including the CARD domain shown to bind to pro-caspase-1.

Regarding the quantity of experimentation, the Office Action on page 9 asserts that dozens of patients in each of the many hundreds of different possible disease states would need to be subjected to a collection of samples for analysis of their DNA in order to acquire statistically significant evidence of an association with a disease or other utility. As discussed above, Applicants respectfully submit that the specification provides sufficient description and guidance for the claimed nucleic acid molecule encoding CLAN-A (SEQ ID NO:97), including binding of the CARD of CLAN to pro-caspase-1 and the teaching in the specification that CLAN-A can trigger pro-caspase-1 activation and is an IL-1β regulator (page 16, lines 21-22 and lines 24-27; page 123, lines 27-29). Accordingly, Applicants respectfully submit that the amount of experimentation asserted in the Office Action to be required is beyond that which is necessary for the claimed nucleic acid molecule encoding CLAN-A (SEQ ID NO:97).

For the reasons of record and those discussed above, Applicants respectfully submit that the specification provides sufficient description and guidance to enable the claimed nucleic acid molecule encoding SEQ ID NO:97 (CLAN-A). Accordingly, Applicants respectfully request that this rejection be withdrawn.

In light of the remarks herein, Applicants submit that the claims are now in condition for allowance and respectfully request a notice to this effect. The Examiner is invited to call the undersigned agent if there are any questions.

To the extent necessary, a petition for an extension of time under 37 C.F.R. 1.136 is hereby made. Please charge any shortage in fees due in connection with the filing of this paper, including extension of time fees, to Deposit Account 502624 and please credit any excess fees to such deposit account.

Respectfully submitted,

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CLAN, a Novel Human CED-4-like Gene

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Proteins governing cell death form the basis of many normal processes and contribute to the pathogenesis of many diseases when dysregulated. Here we report the cloning of a novel human CED-4-like gene, *CLAN*, and several of its alternatively spliced isoforms. These caspase-associated recruitment domain (CARD)-containing proteins are expressed at varying degrees in normal human tissues and may contribute to a number of intracellular processes including apoptosis, cytokine processing, and NF-κB activation. The CARD of the CLAN proteins binds a number of other CARD-containing proteins including caspase-1, BCL10, NOD2, and NAC. Once their physiologic functions are uncovered, CLAN proteins may prove to be valuable therapeutic targets.

Key Words: apoptosis, CARD, CED-4, NACHT, LRR, cell death, caspases

INTRODUCTION

Apoptosis is a highly regulated program of cell suicide that has important roles in the normal development of multicellular organisms, maintenance of tissue homeostasis, and elimination of potentially harmful cells from the body. During mammalian embryonic development, apoptosis governs the proper formation of limbs and digits through the control of mesodermal tissue loss [1], the development of the nervous system [2], and the elimination of self-recognizing B cells and T cells [3]. Alterations in the regulatory components of the apoptotic machinery contribute to many diseases, including autoimmunity, AIDS, stroke, heart failure, neurodegenerative disorders, and cancer [4].

The core components of the apoptotic machinery are evolutionarily conserved and many were originally discovered in the nematode *Caenorhabitis elegans*. One of these required components, CED-4, is critical in coupling cell death stimuli to the activation of dormant proteases known as "caspases" [5]. Proteins within the CED-4 family are characterized by a nucleotide-binding oligomerization domain (NB-ARC, or NACHT domain) and a caspase-associated recruitment domain (CARD), which binds the pro-forms of certain caspases.

We discovered the coding sequence for a novel CED-4-like protein through a bioinformatics approach using public databases. Homology screening found a genomic region on chromosome 2 that was predicted to contain both a NACHT domain and a CARD domain. Corresponding cDNAs were cloned, verifying that this gene is expressed. We have designated this gene *CLAN* (for CARD, LRR, and NACHT-containing protein) because at least one of the proteins it encodes contains CARD, leucine-rich repeat (LRR), and NACHT domains. Here we report the molecular cloning of cDNAs encoding CLAN and several smaller isoforms of this protein that are differentially expressed in human tissues. All four isoforms contain a CARD motif capable of associating with several other CARD-containing proteins, including pro-caspase-1.

RESULTS

Cloning of *CLAN* cDNAs

We searched the HTSG database of human genomic DNA sequence data for regions capable of encoding CARDs using the amino acid sequence of the cellular inhibitor of apoptosis protein-1 (cIAP1) as a query with the TBLASTn method. A genomic locus on human chromosome 2p21–p22 was thus identified. This locus was not recognized in the human genomic database and was not previously annotated. Using GENESCAN for exon prediction, additional regions potentially encoding a NACHT domain and regions corresponding to LRR domains were also recognized 3′ to the potential CARD-encoding sequences, suggesting the presence of a CED-4-like gene. Several partial EST clones have since appeared in the NCBI database (acc. nos. AV719179, AI263294, AV656315, AW337918, BF207840, AW418826, BK903662, AI023795, H25984), none of which contain the *CLAN* CARD region.

Gene-specific primers based on the genomic sequence data were used in conjunction with two universal primers to

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EXHIBIT 1

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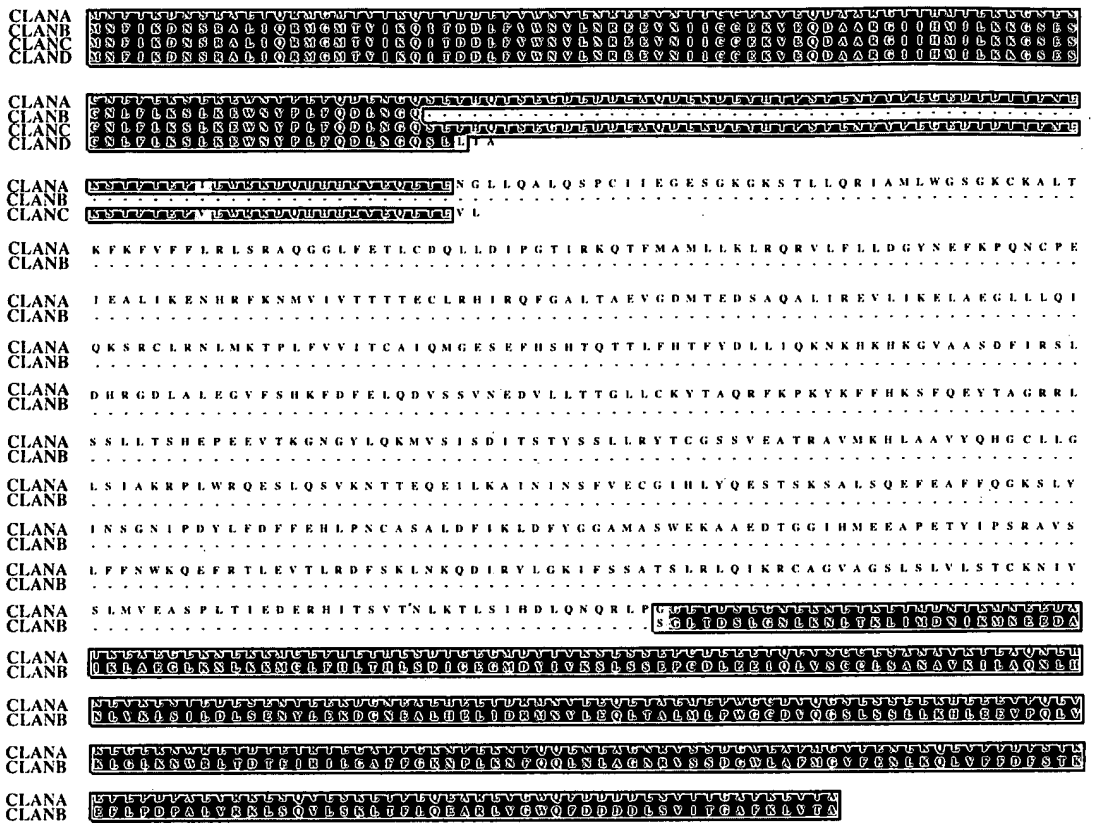

FIG. 1. Clustal-W alignment of the amino acid sequence of the four CLAN splice variants. Identical residues are shaded darker; conserved amino acid changes are shaded lighter.

isolate several cDNAs from human liver and lung RNA sources (Fig. 1). The longest transcript, termed *CLAN-A*, is 3.370 kb with an open reading frame (ORF) encoding a 1024 amino-acid protein containing CARD, NACHT, and LRR domains, as well as a predicted sterile α-motif (SAM) domain. A second transcript, termed *CLAN-B*, is 1.374 kb, with an ORF encoding a 359 amino-acid protein containing an identical CARD directly spliced to the LRRs. *CLAN-C*, the third transcript isolated, is 0.768 kb and encodes a 156 amino-acid protein containing just the CARD and an additional region lacking homology to recognizable domains. Finally, the shortest transcript found, *CLAN-D*, is 0.578 kb and contains an ORF encoding a 92 amino-acid protein encompassing only the CARD followed by 9 amino acids.

Comparisons of these cDNA sequence data with the genomic DNA sequence data found in the HTSG database indicated that *CLAN* consists of 12 exons, spanning 41.3 kb on chromosome 2p21–p22 (Fig. 2A). Three differences were found between the sequence of the *CLAN* cDNA and the sequence within the public database. Also, the region encompassing the first 12 nucleotides of CLAN (5′ UTR) does not have an equivalent fragment in the public database. Two different transcriptional start sites are used (corresponding to the beginning of either exon 1 or 2), but both are spliced to exon 3 at the beginning of the CARD. Exons 6 and 7 contain additional internal splice donor sites used to generate *CLAN-C*. mRNA splicing events are predicted to give rise to the *CLAN-A*, *CLAN-B*, *CLAN-C*, and *CLAN-D*

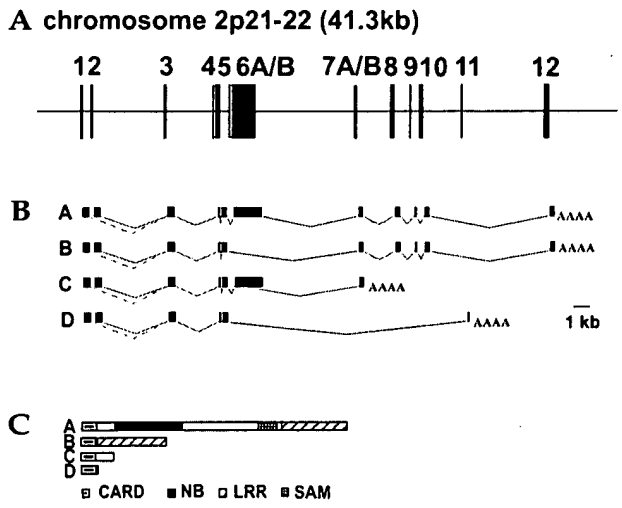

FIG. 2. Intron/exon organization of *CLAN*. (A) Genomic organization of *CLAN* on chromosome 2 deduced from the BAC 164M19 sequence from the SPG4 candidate region at 2p21–2p22 (acc. no. AL121653) and *Homo sapiens* chromosome 2 working draft sequence (acc. no. NT_005194.1). (B) mRNA splicing generates *CLAN-A*, *CLAN-B*, *CLAN-C*, and *CLAN-D*. (C) Deduced domain structure for the splice variants.

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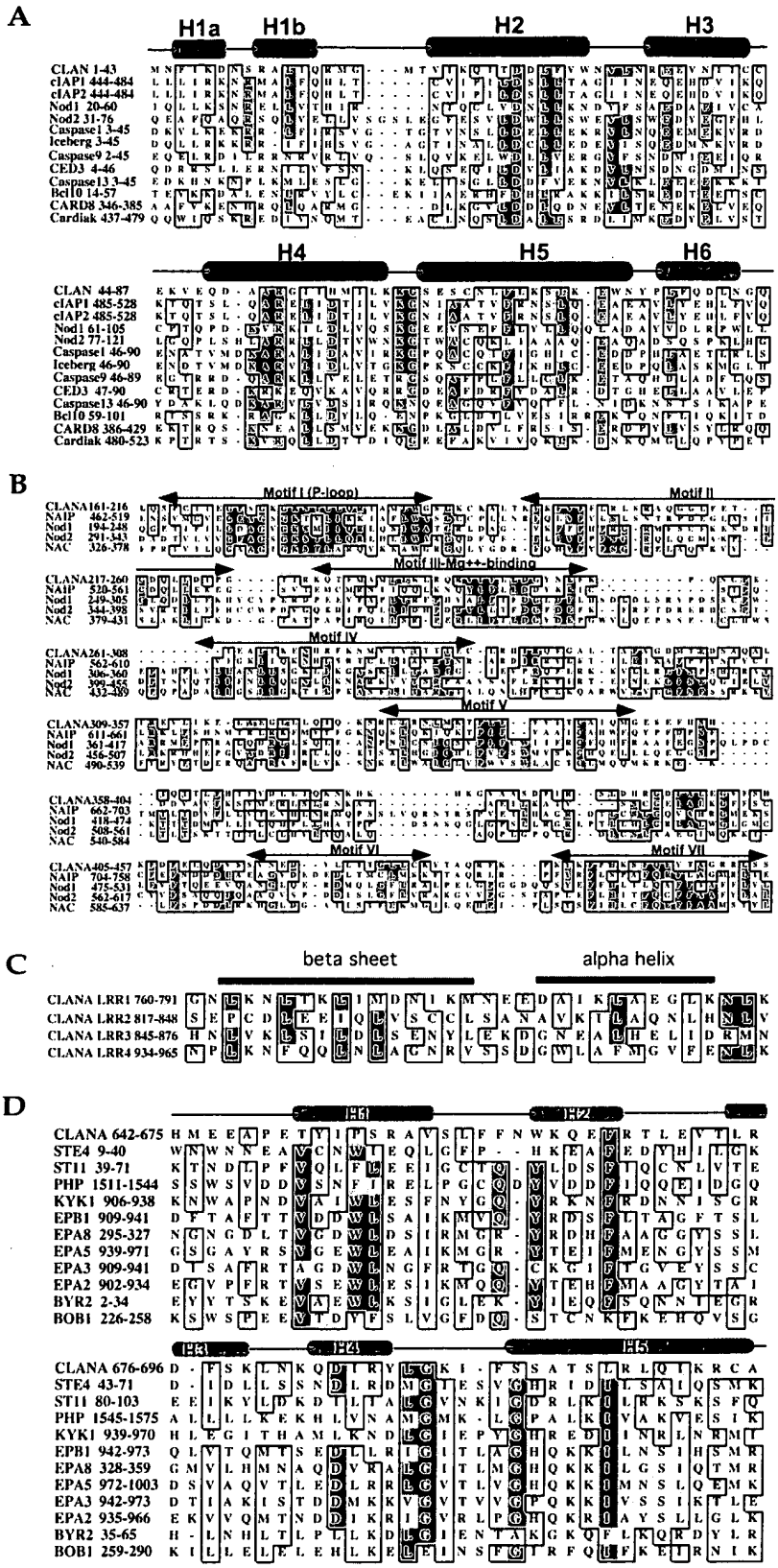

FIG. 3. Domain alignments in CLAN. (A) Predicted amino-acid sequence of CLAN CARD and alignment with the most similar CARDs of other proteins. Top, the predicted secondary structure for the CLAN CARD, as predicted from the solved structure of ICEBERG. Accession numbers are as follows: cIAP1, Q9UP46; cIAP2, Q13489; NOD1, Q9Y239; NOD2, AAG33677; caspase-1, Q9TV13; ICEBERG, P57730; caspase-9, Q9IB63; CED3, P45436; caspase-13, O75601; BCL10, CAA06955; CARD8, AAG50014; CARDIAK, AAC25668. (B) Alignment of the NACHT domain of CLAN with most homologous NACHT domains from NAIP, NOD1, NOD2, and NAC. Shown are the seven conserved boxes including the Walker A and B box. Accession numbers are as follows: NAIP, AAC62261; NOD1, Q9Y239; NOD2, AAG33677; NAC, AAK00748. (C) Alignment of the four LRRs found in CLAN. Top, the secondary structure as predicted from the known structure of the human placental ribonuclease/angiogenin inhibitor. (D) Alignment of the SAM domain found in CLAN with the most similar known SAM domains. At top is shown the secondary structure as predicted from the solved structure of the Eph receptor SAM domain. Identical residues are shaded darker, conserved amino acid changes are shaded lighter. Accession numbers are STE4 (X61924), ST11 (P23561), PHP (P39769), KYK1 (P18160), EPB1 (P54762), EPA8 (P29321), EPA5 (P54755), EPA 3 (P29318), EPA 2 (P29317), BYR2 (P28829), and BOB1 (P38041), respectively.

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transcripts and encoded proteins (Fig. 2B). All the exon/intron splice junctions follow the conserved GT/AG consensus rule.

As predicted by SMART (EMBL, Heidelberg, Germany), CLAN contains a CARD. A ψ-BLAST search of the non-redundant database using the CLAN CARD as query identified several homologous CARDs, including those from cIAP1 and -2 (58%), caspase-1 and ICEBERG (50%), NOD1, NOD2, and CARD8 (~38%) and caspase-13, CED3, caspase-9, BCL10 (CIPER) and CARKIAK/RIP2 (~30%; Fig. 3A). Following the CARD, we observed a domain containing a consensus sequences for Walker A (GXXXXGKT/S) and B boxes (V/LI/V/LV/IL/VDS) [6] and additional characteristics of the newly founded family of NTPases termed the NACHT family [7]. By ψ-BLAST search, the NACHT domain of CLAN shows highest similarity to the NACHT domain of NAIP (60%), followed by NOD1 (49%) and NOD2 (47%; Fig. 3B). LRR domains are also found near the carboxy terminus of the A and B isoforms of the protein (Fig. 3C). The C-terminal end consists of four repeated LRRs, each containing a predicted β-sheet and α-helical structure, which is in agreement with the prototypical horseshoe-shaped structure of LRRs [8]. LRR 1 represents a non-Kobe and Deisenhofer (non-K/D) LRR of the structure XaXXaXaX(N/C/T/Q)±Xa (where "X" is any amino acid and "a" represents an aliphatic amino acid), whereas LRRs 2, 3, and 4 are in accordance with Kobe and Deisenhofer (K/D) LRR with the consensus sequence XaXXaXaXX(N/C/T/Q)±Xa. LRR 2 also shares sequence homology to a prototypical ribonuclease inhibitor type A (RI type A). By ψ-BLAST searches the LRRs show 49% sequence identity to the placental ribonuclease/angiogenin inhibitor (RAI; Fig. 3C). Sequences between the NACHT and LRR domains show some similarity to the SAM, a domain consisting of five α-helices, originally found in proteins involved in developmental processes. The SAM domain has been shown to function as a protein–protein interaction domain, with ability to homooligomerize as well as hetero-oligomerize with other SAMs [9] (Fig. 3D).

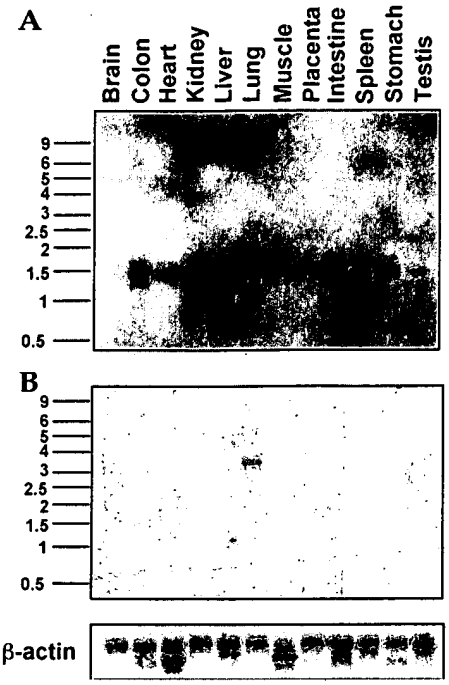

FIG. 4. Expression of *CLAN* mRNA in normal human tissues. Northern membranes composed of normalized poly(A)$^+$ RNA from human tissues were hybridized with a labeled probe corresponding to the CARD of *CLAN* (A) or to the NACHT and LRR of *CLAN* (B). As a loading control, blots were reprobed with a cDNA corresponding to β-actin. Molecular weight markers are indicated in kilobase-pairs (kbp).

In vivo expression of CLAN

We examined which of the splice variants of *CLAN* are expressed in adult human tissues. Northern blot analysis using the CARD of *CLAN* as a probe revealed expression of an approximately 1.5-kb transcript corresponding to *CLANB* in nearly all tissues examined with highest expression in lung and spleen (Fig. 4A). The *CLAN-A*, *CLAN-C*, and *CLAN-D* splice variants are not detected on this blot, probably due to lower expression levels. Northern blot analysis using the NACHT and LRR of *CLAN-A* as a probe revealed expression of an approximately 3.5-kb mRNA corresponding to *CLAN-A*, primarily in the lung (Fig. 4B).

To further explore the tissue-specific patterns of expression of *CLAN* splicing variants, RT-PCR assays were devised which are specific for the *CLAN-A*, *CLAN-B*, *CLAN-C*, and *CLAN-D* isoforms. RT-PCR analysis showed that *CLAN-B* was present throughout human tissues, consistent with the northern blot analysis (Fig. 5). In contrast, *CLAN-A* was restricted to lung, colon, brain, prostate, spleen, and leukocytes. Further analysis of leukocyte sub-populations revealed expression of the *CLAN-A* isoform predominantly in the monocyte cell fraction, with lower expression found in granulocytes and no expression in lymphocytes. Though these PCR assays were not carried out in a quantitative fashion, PCR amplification of *CLAN-A* was at the highest levels in the lung, which is in agreement with the northern blot results. Expression of *CLAN*-C was absent from all normal tissues tested, but expression was evident in the cell line HEK293T (data not shown), indicating this transcript can be produced under some circumstances. *CLAN-D* transcripts were detected only in brain by RT-PCR.

CLAN protein interactions

As the CARD is a homophilic interaction domain [10], we investigated which of the known CARD proteins can bind the CARD of CLAN, thus providing hints about possible functions of the CLAN proteins. The CARD of CLAN was expressed as an epitope-tagged protein in HEK293T cells in co-transfections with a variety of other epitope-tagged CARD-containing proteins, and lysates derived from these cells were used for co-immunoprecipitation assays (Fig. 6). The CARD of CLAN bound readily to full-length pro-caspase-1, but did not bind another CARD-containing caspase, caspase-9. Among the other CED-4 family members which contain a CARD in conjunction with a nucleotide-binding

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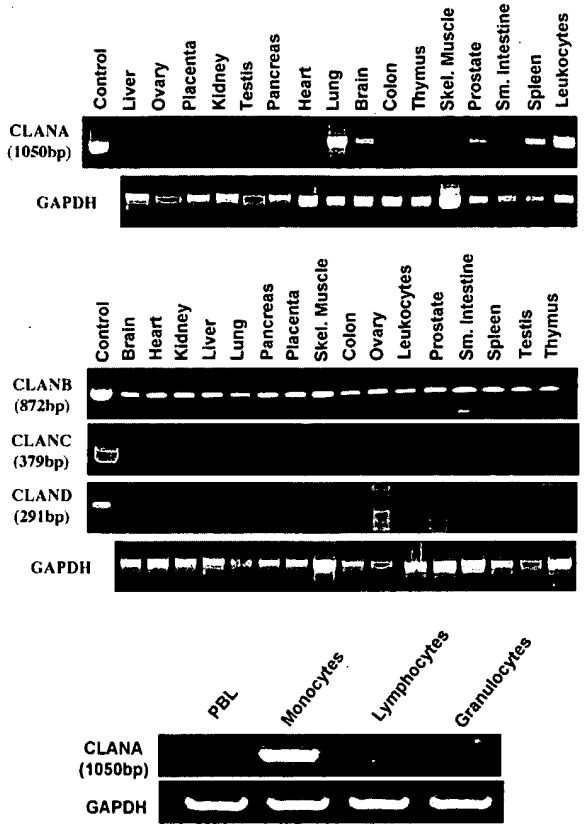

FIG. 5. RT-PCR analysis of *CLAN* expression. cDNAs derived from multiple human tissues were subjected to PCR using *CLAN* isoform-specific primers. In addition, circulating blood leukocytes were fractionated and cDNA was prepared for PCR analysis. As a control for message integrity, PCR for GAPDH was also carried out. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining with UV illumination.

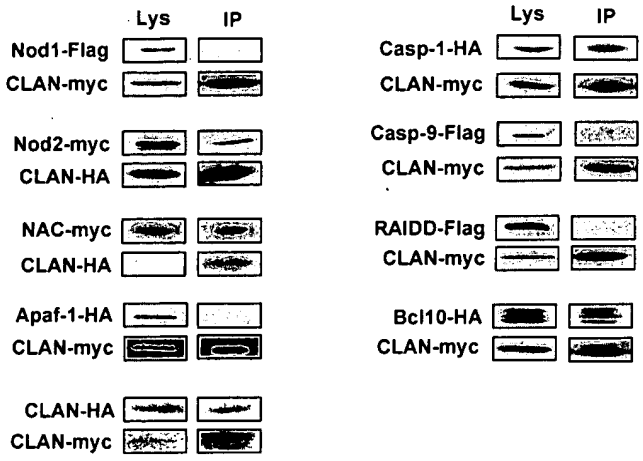

FIG. 6. Identification of CLAN interaction partners by co-immunoprecipitation. The CARD common to all CLAN isoforms was co-expressed as an epitope-tagged protein in HEK293T cells together with other epitope-tagged CARD-containing proteins by transient transfection. Immune complexes (IP) were separated by 12–15% SDS-PAGE and subjected to immunoblotting with antibodies corresponding to each epitope. As a control, 10% of the input cell lysate (Lys) was loaded directly in gels.

domain, CLAN interacted with the CARDs of NOD2 and NAC, but not with Apaf-1 or NOD1. Finally, the CLAN CARD was found to associate with BCL10, but not with another adapter protein, RAIDD.

Discussion

Here we have described a novel human gene, *CLAN*, capable of producing transcripts encoding several CARD-containing proteins. The predicted CLAN protein contains three domains ordered in a manner similar to that seen in NOD1 and NOD2 [11,12]: a CARD domain located near the amino terminus, followed by a central NACHT domain and then a span of LRR near the C terminus. Unlike NOD1 and NOD2, however, a putative SAM domain is also found in this protein. The NACHT domain contains a P-loop region that has previously been shown to be important for nucleotide-dependent self-oligomerization. This structural feature is also shared with plant disease resistance "R" proteins. Similar to the Ced-4 homologues of animals, these plant proteins contain N-terminal effector domains linked to a NACHT domain followed by multiple LRR repeats. These proteins mediate defense responses in plants to pathogens [13]. The expression of the longest isoform, CLAN-A, seems to be limited primarily to lung and also to monocytes, where another CED-4 homologue, NOD2, has been reported to be exclusively expressed [12].

The best characterized mammalian CED-4 homologue, APAF-1, recruits pro-caspase-9 (an initiator caspase) into a protein complex in response to mitochondrial cytochrome *c* release [14], thus initiating the proteolytic cascade and resulting in apoptosis. In addition to apoptosis, CARD-containing proteins have also been shown to have other effects on cellular functions including cytokine processing and NF-κB induction. Some CED-4-like proteins, such as NOD1 (CARD4) and NOD2, activate NF-κB through interactions with another CARD-containing protein, CARDIAK (RIP2/RICK), which in turn binds to NEMO (IKKγ), a component of the IKB-kinase complex [11,12,15–17]. The LRRs of NOD1 and NOD2 have recently been shown [18] to function in a manner analogous to plant "R" gene products in that they act as cytosolic receptors (either directly or indirectly) for lipopolyscharides produced by invading bacteria, thus providing a trigger for NF-κB activation and subsequent immune responses. In addition to these CED-4-like proteins, CARD-containing proteins without NACHT and LRR domains are also known to induce NF-κB through direct binding to BCL10 or CARDIAK [19–21].

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As evidenced by their interactions with other CARD proteins, the novel isoforms of CLAN described here may have an influence on apoptosis, cytokine processing, or NF-κB activity. Interactions of CLAN with pro-caspase-1 can be indicative of a role for CLAN as an interleukin-1β regulator. In this regard, different isoforms of CLAN would be predicted to have opposing effects on pro-caspase-1 activation. The longest isoform, CLAN-A, for example, might trigger pro-caspase-1 activation by the "induced proximity" mechanism as a result of oligomerization mediated by its NACHT domain. In contrast, the shorter isoforms of CLAN lacking this self-oligomerization would be expected to operate as competitive antagonists of pro-caspase-1 activation, analogous to ICEBERG, a CARD-containing protein that competes with CARDIAK (RIP2/RICK) for binding to pro-caspase-1 [21]. Tissue-specific differences in the expression of CLAN-A and shorter CLAN isoforms thus may have a profound effect on pathways involved in pro-caspase-1 activation and inflammatory responses. Interactions of CLAN with NAC also suggest this protein may have an influence on apoptosis mediated by APAF-1, as NAC binds APAF-1 and enhances its ability to activate caspase-9 in response to cytochrome *c* [22]. Finally, CLAN associations with NF-κB regulators such as BCL10 and NOD2 suggest that at least some of the CLAN isoforms may influence the activity of this transcription factor. It is also notable that the CLAN locus lies in close proximity to the spastin gene (on chromosome 2p21–22), encoding a AAA protein, which is frequently mutated in autosomal dominant hereditary spastic paraplegia (AD-HSP) [23]. The CLAN locus is found on the strand opposite the SPG4 (SPAST) locus, but with no overlapping regions. It has yet to be determined whether mutations in CLAN occur in patients with this neurodegenerative disorder.

The physiological functions of the isoforms of CLAN remain to be delineated. But if it is found to have a regulatory role in the processes of inflammation or cell death, CLAN may prove to be a valuable pharmacological or genetic target for the treatment of a variety of illnesses.

Materials and Methods

Bioinformatics. The *CLAN* cDNA was found using the HTGS database, performing searches with TBLASTn using the CARD sequence of cIAP1 as query. This search revealed strong homology with a human genomic clone (acc. no. AQ889169).

cDNA cloning. *CLAN*-specific primers corresponding to sequences within the putative CARD and NACHT regions (as determined from genomic DNA sequence data) were used in conjunction with two universal primers to isolate *CLAN* cDNAs from first-strand liver and lung cDNA by nested PCR according to the manufacturer's protocol (SMART RACE, Clontech). Primers used for amplification are 5′ RACE primers (5′-CATGTGAATGATCCCTCTAGCAG-3′; nested 5′-GGGCTCGGCTATCGTGCTCTA-3′) and 3′ RACE primers (5′-ACGATAGCCGAGCCCTTATTC-3′; nested 5′-GTATGGAATGTTCTGAATCGC-3′). Amplification products were purified from agarose gels, ligated into the TA cloning vector (Promega), and sequenced. Four ORFs were deduced and multiple clones of each isoform were sequenced to ensure fidelity of PCR products.

Plasmid construction. A cDNA encoding the *CLAN* CARD domain was amplified using PFU polymerase and specific primers (5′-CCCGGATCCATGAATTTCATAAAGGACAATAGC-3′, 5′-CCCTTCGAACAAGTCCTGAAATAGAGGATA-3′) containing *Bam*HI and *Hin*dIII sites. The resulting PCR product was ligated into pcDNA3.1(-)/Myc-His_6 A (Invitrogen) which places the myc-His_6 tag at the C terminus of expressed proteins. pcDNA3/HA-CLAN (CARD) was created using a similar strategy. Authenticity of all vectors was confirmed by DNA sequencing.

RT-PCR. Total RNA was isolated from cells using Trizol reagent (BRL) and 2 μg was used to generate cDNA in a reverse transcription reaction with Superscript II (BRL). PCR was carried out in an Eppendorf thermal cycler using *Taq* polymerase (BRL) and the following isoform-specific primer pairs: *CLAN-A*, 5′-GGTGGAGCAGGATGCTGCTAGAGG-3′, 5′-CACAGTGGTCCAGGCTCCGAATGAAGTCA-3′; *CLAN-B*, 5′-CATCATTTGCTGCGAGAAGGTGGAG-3′, 5′-TTAACTTGGATAACACTTGGCTAAG-3′; *CLAN-C*, 5′-GTAAACATCATTTGCTGCGAGAA-3′, 5′-CCCGGGCAGGTAGAAGATGCTAT-3′; *CLAN-D*, 5′-AATTTCATAAAGGACAATAGCCGAG-3′, 5′-TGTCTACTGTACTTTCTAAGCTGTT-3′. For determination of *CLAN* isoform expression in normal human tissues, we used a panel of cDNA specimens derived from various human tissues (Clontech). Peripheral blood leukocytes were obtained from heparinized venous blood by Ficoll-Paque (Amersham) density-gradient centrifugation. Red blood cells were removed from granulocytes by short incubation in hypotonic lysis buffer. Monocytes were separated from lymphocytes by adherence to plastic dishes. Cells were lysed in Trizol and RNA subjected to RT-PCR as described.

RNA blots. Hybridization probes corresponding to the common CARD domain of all 4 *CLAN* isoforms or the NACHT and LRR regions were radiolabeled by random priming with hexanucleotides (Roche) and [α-^{32}P]-dCTP, or digoxigenin-labeled with a commercially available kit (Roche), incubated with blots containing human poly(A)$^+$ RNA derived from various human tissues (Origene), washed at high stringency, and exposed to X-ray film. Positive signals were detected by autoradiography or by immunoblotting with HRP-conjugated anti-DIG antibody and an enhanced chemiluminescence method (ECL; Amersham).

Co-immunoprecipitation assays. HEK293T cells were seeded onto six-well plates (35-mm wells) and transfected with 0.2–2 μg plasmid DNA using Superfect (Qiagen) after 24 h. After culturing for 1 d, cells were collected and lysed in isotonic lysis buffer (142.4 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES, pH 7.4, 0.5 mM EGTA, 0.2% NP-40, 12.5 mM β-glycerophosphate, 2 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and 1× protease inhibitor mix (Roche)). Lysates were clarified by centrifugation and subjected to immunoprecipitation using agarose-conjugated anti-c-myc antibodies (Santa Cruz), or non-specific control antibodies and Protein G-agarose for 2–24 h at 4°C. Immune complexes were washed 4 times with lysis buffer, boiled in Laemmli buffer, and separated by 12–15% PAGE. Immune complexes were then transferred to PVDF membranes and immunoblotted with anti-c-myc (Santa Cruz), anti-HA (Roche), or anti-Flag (Sigma) antibodies. Membranes were washed, incubated with HRP-conjugated secondary antibodies, and reactive proteins were detected using ECL.

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Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession numbers AY027787 (CLAN-A), AY027788 (CLAN-B), AY027789 (CLAN-C), and AY027790 (CLAN-D).

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Multiple Roles of CLAN (Caspase-Associated Recruitment Domain, Leucine-Rich Repeat, and NAIP CIIA HET-E, and TP1-Containing Protein) in the Mammalian Innate Immune Response[1]

Jason S. Damiano, Ruchi M. Newman, and John C. Reed[2]

NAIP CIIA HET-E and TP1 (NACHT) family proteins are involved in sensing intracellular pathogens or pathogen-derived molecules, triggering host defense responses resulting in caspase-mediated processing of proinflammatory cytokines and NF-κB activation. Caspase-associated recruitment domain, leucine-rich repeat, and NACHT-containing protein (CLAN), also known as ICE protease-activating factor, belongs to a branch of the NACHT family that contains proteins carrying caspase-associated recruitment domains (CARDs) and leucine-rich repeats (LRRs). By using gene transfer and RNA-interference approaches, we demonstrate in this study that CLAN modulates endogenous caspase-1 activation and subsequent IL-1β secretion from human macrophages after exposure to LPS, peptidoglycan, and pathogenic bacteria. CLAN was also found to mediate a direct antibacterial effect within macrophages after *Salmonella* infection and to sensitize host cells to *Salmonella*-induced cell death through a caspase-1-independent mechanism. These results indicate that CLAN contributes to several biological processes central to host defense, suggesting a prominent role for this NACHT family member in innate immunity. *The Journal of Immunology*, 2004, 173: 6338–6345.

Host-pathogen interactions play important roles in many human diseases. A hyperinflammatory host response to bacterial cell wall components such as LPS is believed to form the basis for many of the pathologic features of sepsis, including localized tissue damage and organ failure (1). This condition afflicts >500,000 people annually in the United States alone and, with mortality rates of 40–70%, represents the leading cause of death within intensive care units (2, 3). Similarly, inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease, are believed to result from defective host response to intestinal bacteria, affecting ~6 of 100,000 and 8.4 of 100,000 persons in the United States, respectively (4). Understanding the biology of the mammalian innate immune response, therefore, is key to developing new treatments for a variety of infectious and inflammatory diseases as well as devising more effective adjuvants for vaccines.

Much is now known about the biological roles and signal transduction mechanisms of TLRs, a highly conserved family of pathogen-sensing, cell surface proteins required for effective innate immune responses (5). These proteins typically contain extracellular spans of leucine-rich repeats (LRRs),[3] which function as receptors for conserved microbial structures and also possess intracellular Toll/IL-1 receptor (TIR) domains that transduce proinflammatory signals (5). For example, TLR4 recognizes bacterial LPS in a complex with CD14 and MD-2, triggering its intracellular TIR-dependent association with MyD88 and leading to activation of NF-κB-dependent gene transcription (6). Macrophage-expressed CD14 is also responsible for internalizing LPS into the cytoplasm, where it may be presented to other intracellular proteins that also contain bacterial-recognition motifs (7, 8).

Among the candidates for sensing intracellular pathogens or pathogen-derived molecules are the NAIP CIIA HET-E and TP1 (NACHT) family proteins, a group of phagocyte-expressed proteins containing spans of LRRs and a characteristic nucleotide-binding domain known as NACHT. Although many details are lacking, NACHT family proteins appear to induce proinflammatory signaling events in response to invading pathogens, similar to the intracellular LRR-containing pathogen resistance proteins found in plants (9). Mutations within the genes encoding human NACHT family proteins have recently been implicated in susceptibility to several chronic hyperinflammatory disorders, including Crohn's disease, Blau syndrome, and Muckle-Wells syndrome, thus providing new possible candidates for pharmacological intervention in these diseases (10–12). Proteins of the NACHT family appear to function independently of the TLRs and typically reside within the cytosol of macrophages and intestinal epithelial cells, which are among the first to encounter invading pathogens. NACHT proteins lack a TIR domain, but exhibit a conserved domain architecture that includes C-terminal LRRs, a central nucleotide-binding NACHT domain, and either an N-terminal caspase-associated recruitment domain (CARD) or a PYRIN domain that links bacterial pattern recognition to other effector proteins, such as procaspase-1 and the Iκ kinase-binding protein RIP2/CARDIAK. Caspase-1 and RIP2/CARDIAK, in turn, are critical for the activation of proinflammatory cytokines such as pro-IL-1β (IL-1β) and for the induction of NF-κB, respectively (13, 14). The activation of NACHT family proteins is thought to

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[3] Abbreviations used in this paper: LRR, leucine-rich repeat; CARD, caspase-associated recruitment domain; ECFP, enhanced cyan fluorescent protein; LDH, lactate dehydrogenase; LTA, lipoteichoic acid; MOI, multiplicity of infection; NACHT, NAIP CIIA HET-E and TP1; PAMP, pathogen-associated molecular pattern; PGN, peptidoglycan; RNAi, RNA interference; SCR, scrambled CLAN sequence; TIR, Toll/IL-1 receptor; CLAN, CARD, LLR, and NACHT-containing protein.

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involve oligomerization via their corresponding NACHT domains, triggered by binding of specific pathogen-derived molecules to the LRRs (15, 16).

A subset of CARD-containing proteins within the greater NACHT family shares considerable amino acid sequence similarity and includes Nod1/CARD4, Nod2/CARD15, and CARD, LRR, and NACHT-containing protein (CLAN), also known as ICE protease-activating factor (17–20). Interest in this branch of the NACHT family has propagated since the discovery of a correlation between hereditary mutations in the Nod2/CARD15 gene and susceptibility to inflammatory bowel disorders such as Crohn's disease and Blau syndrome (10, 11). Additional research has identified muramyl dipeptide, the minimal bioactive peptidoglycan motif common to all bacteria, as the ligand recognized nonredundantly by the LRRs of Nod2 (21). The inactivating truncation mutations found within the LRR domain of Nod2 have been hypothesized to contribute to defective NF-κB-initiated inflammatory responses in the gut after exposure to invasive bacteria. Although much emphasis has been placed on the importance of Nod2 in the innate immune response to specific bacterial cell wall components, this protein appears to play a nonessential or redundant role in host defense, as evidenced by a knockout mouse model (22, 23). However, deletion of the murine Nod2 gene did provide limited protection against LPS-induced death of mice, suggesting a contributing role in sepsis (23). Similarly, Nod1 has been shown to recognize a unique muropeptide found in Gram-negative bacteria (22, 24), but a critical role in host defense has not yet been established for this protein. It remains to be determined whether other NACHT family proteins with similar domain architectures are essential for bacterial pattern recognition and inflammatory responses within macrophages and other host defense cells.

The Nod1/Nod2-like protein CLAN (also known as ICE protease-activating factor or CARD12) was originally described as a CARD, LRR, and NACHT-containing protein capable of binding and activating procaspase-1 after overexpression in HEK293T cells (17, 25). The function of CLAN in human macrophages (where it is endogenously expressed) has not been reported previously, nor have the specific ligands responsible for its activation been identified. In this study we used gene transfer and RNA interference (RNAi) to explore the functions of CLAN in the human monocytic cell line THP-1, differentiated into macrophages by PMA. Our findings demonstrate that CLAN modulates IL-1β production induced by LPS, peptidoglycan (PGN), and invasive bacteria, and also controls an antimicrobial mechanism for reducing accumulation of intracellular bacteria in macrophages. In addition to these pathogen-derived responses, CLAN is capable of inducing the death of macrophages after their infection by high amounts of bacteria. Thus, CLAN regulates several events relevant to effective host defense mechanisms against invading bacteria.

Materials and Methods

Cell lines and bacterial strains

The THP-1 monocytic leukemia cell line was obtained from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 supplemented with 10% FCS, 1% (v/v) penicillin (100 U/ml), streptomycin (100 U/ml), and L-glutamine. Cells were maintained at 37°C in a 5% CO_2-95% air atmosphere and were subcultured every 6 days. *Salmonella enteritidis* strain LK5 was a gift from Dr. S. Maloy (San Diego State University, San Diego, CA); *Salmonella typhimurium* strain SL1344, *S. typhimurium* SL1344 SipB$^-$ and *Shigella flexneri* were gifts from Dr. S. Falkow (Stanford University, Stanford, CA).

Generation of stably transfected THP-1 cell lines

The pLXRN retroviral vector (BD Pharmingen, San Diego, CA) containing a neomycin resistance cassette and a cDNA encoding full-length CLAN (with a C-terminal c-Myc tag) or empty pLXRN was transfected along with a vector encoding the stomatits virus glycoprotein (VSV-G) into the 293-GP packaging cell line using Superfect (Qiagen, Valencia, CA). At 24 h post-transfection, virus-containing supernatants were collected, passed through 0.45-μm pore size, syringe-top filters, and applied to 5×10^5 THP-1 cells in the presence of 5 μg/ml polybrene in 24-well plates. Cells were sedimented by centrifugation for 45 min at $800 \times g$. After three rounds of infection, cells were incubated at 32°C for 24 h and provided fresh medium containing 800 μg/ml geneticin the following day. To generate CLAN-deficient monocytes, oligonucleotides encoding a short RNAi hairpin construct specific for the CLAN coding sequence were subcloned into pSuppress (gift from D. Billadeau, Mayo Clinic, Rochester, MN), an RNAi-expressing plasmid based on pSuper, containing the RNA Pol III H1 promoter. Annealed oligonucleotides were cloned upstream of the RNA polymerase III promoter (primer 1 sequence, 5′-GATCCCCGAAGCAGA CATTCATGGCCTTCAAGAGAGGCCATGAATGTCTGCTTCTTTTTG GAAA-3′; primer 2 sequence, 5′-AGCTTTTCCAAAAAGAAGCAGA CATTCATGGCCTCTCTTGAAGGCCATGAATGTCTGCTTCGGG-3′). The cassette containing the promoter and CLAN sequence was then subcloned into pLXRN, and the sequence was verified and used to create packaged virus as described above. A THP-1 cell line stably expressing a control RNAi hairpin (scrambled CLAN sequence (SCR)) was created similarly. The levels of vector-derived CLAN mRNA expression in infected THP-1 cells were determined by RT-PCR analysis (in the linear range of amplification) using conditions described previously (17). The expression of CLAN protein in infected THP-1 cells was also confirmed by immunoblotting using an Ab specific for the c-Myc epitope tag, as previously described (17).

Macrophage infections

S. enteritidis strain LK5 and mouse-virulent *S. typhimurium* strain SL1344 were grown standing in high salt Luria Bertoni broth overnight until mid-log growth phase was achieved. *S. flexneri* was grown overnight in tryptic soy broth, then subcultured 1/50 for 2 h before infection. THP-1 cells (3×10^5) were differentiated for 16 h with PMA (50 ng/ml), washed, and exposed to bacteria at a multiplicity of infection (MOI) of 5–50 for 1 h in antibiotic-free RPMI 1640 containing 5% FCS at 37°C. Cells were washed in HBSS, then fresh medium containing gentamicin (100 μg/ml) was added for 1 h to kill extracellular bacteria. After supernatant collection and extensive washing, cells were incubated for additional time periods in medium containing 10 μg/ml gentamicin, after which they were lysed in 1% Triton X-100 to release intracellular bacteria. Serial dilutions of lysates were spread on Luria Bertoni-agar plates and incubated overnight at 37°C to assess levels of viable intracellular bacteria. Cytotoxicity was evaluated by measuring cytosolic lactate dehydrogenase (LDH) release into the supernatant using a colorimetric assay (CytoTox96; Promega, Madison, WI). Secreted IL-1β levels were analyzed using an ELISA (BD Pharmingen). In some experiments, differentiated cells were preincubated for 1 h with 100 μM of the caspase-1 inhibitor z-WEHD-fmk (Alexis, San Diego, CA), 50 μM of the pan-caspase inhibitor z-VAD-fmk (Calbiochem, La Jolla, CA), or vehicle control (DMSO) before bacterial exposure.

Determination of phagocytic index

To determine whether differences in *Salmonella* uptake accounted for the effect of CLAN on intracellular bacteria levels during gentamicin protection assays, an enhanced cyan fluorescent protein (ECFP)-expressing *S. typhimurium* strain was generated using electrocompetent *S. typhimurium* and pECFP plasmid (Invitrogen Life Technologies, San Diego, CA). THP-1/Neo or THP-1/CLAN cells (10^5) were differentiated on four-well glass chamber slides overnight using PMA, after which the cells were infected for 1 h in antibiotic-free medium at an MOI of 5. Slides were washed extensively in PBS and fixed in 4% methanol-free formaldehyde. THP-1 nuclei were stained with 4′,6-diamidino-2-phenylindole, and 500 cells were scored for the presence of ECFP-expressing *S. typhimurium* for each cell line.

Pathogen-associated molecular pattern (PAMP) exposure

THP-1 cells (3×10^5) were differentiated for 16 h, washed, and exposed to LPS isolated from *Escherichia coli* strain 055:B5 (Sigma-Aldrich, St. Louis, MO; used at 1–1000 ng/ml), PGN from *Staphylococcus aureus* (Fluka, Buchs, Switzerland; used at 0.5–10 μg/ml), lipoteichoic acid (LTA) from *S. aureus* (Fluka; used at 10 μg/ml), or unmethylated CpG DNA oligonucleotides (gift from ISIS Pharmaceuticals; used at 10 μM). In some experiments, cells were preincubated with caspase inhibitors before PAMP exposure, as described. After 6 h, supernatants were collected and analyzed for secreted LDH and IL-1β.

Results

Manipulating CLAN expression levels in THP-1 cells

To study the functions of CLAN, we generated a THP-1 cell line overexpressing CLAN and a THP-1 cell line deficient in endogenous CLAN using retroviral gene insertion of the full-length CLAN cDNA (THP-1/CLAN) or a construct encoding an RNAi hairpin specific for CLAN (THP-1/HP), respectively. As controls, two additional cell lines were created: one expressing only the neomycin resistance gene (THP-1/Neo) and one expressing a nonspecific RNAi hairpin construct corresponding to the scrambled CLAN RNAi sequence (THP-1/SCR). Expression levels of CLAN mRNA were assessed by RT-PCR using control cells (THP-1/Neo) as a reference (Fig. 1*A*). THP-1 cells containing the integrated CLAN expression vector had ~2-fold higher levels of CLAN mRNA than Neo control cells. Conversely, THP-1 cells containing the CLAN RNAi vector expressed ~50% lower levels of CLAN mRNA compared with THP-1/Neo control cells, whereas CLAN expression levels in THP-1/SCR were approximately equivalent to those seen in THP-1/Neo (Fig. 1*A*). The lack of anti-CLAN Abs precluded assessment of the effects of gene transfer and RNAi on levels of CLAN protein, but the expression of the Myc-tagged CLAN protein in retrovirus-infected THP-1 cells was verified by immunoblotting (not shown).

Endogenous CLAN mRNA levels were also analyzed in wild-type THP-1 cells after exposure to LPS, the monocyte-differentiating agents PMA and CSF-1, and proinflammatory cytokines IFN-γ and TNF-α (Fig. 1*B*). In comparison with untreated control cells, CLAN expression was up-regulated at 16 h after stimulation with LPS and TNF-α, suggesting a role for endogenous CLAN in the macrophage responses to these inflammatory mediators. By comparison, PMA had relatively little effect on CLAN mRNA levels in THP-1 cells despite inducing these monocytic leukemia cells to undergo differentiation from small round suspension cells to large adherent macrophages. The cytokines IFN-γ and CSF-1 also had little or no effect on CLAN expression in these experiments.

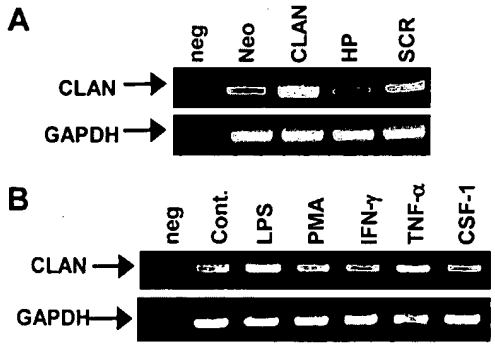

FIGURE 1. Altered CLAN levels in stably transfected and stimulated THP-1 cells. *A*, THP-1 cells were infected with recombinant retroviruses engineered to express full-length CLAN with a C-terminal Myc tag, a RNAi hairpin construct specific for CLAN (HP), or a nonspecific SCR. Polyclonal cell populations were selected with G418, and CLAN mRNA levels were analyzed by RT-PCR using primers that detect both endogenous and retrovirus-induced CLAN mRNA. THP-1 cells stably infected with empty vector and selected in G418 (Neo) served as a control for comparison of CLAN mRNA expression. RT-PCR analysis was performed using 2 μg of RNA, oligo(dT) priming, and primers specific for either CLAN (*top*) or GAPDH (*bottom*). PCR performed without template cDNA (neg) excluded a contribution from contaminating DNA. *B*, Wild-type THP-1 cells were treated for 16 h with LPS (200 ng/ml), PMA (50 ng/ml), IFN-γ (1000 U/ml), TNF-α (50 ng/ml), or CSF-1 (20 ng/ml). RNA was isolated, and 2 μg was analyzed by RT-PCR, using primers specific for either CLAN (*top*) or GAPDH (*bottom*).

The increase in the expression of CLAN mRNA appears to represent a relatively late response to these proinflammatory factors, because no alterations in expression were observed 4 h after stimulation (data not shown).

CLAN-dependent IL-1β secretion in macrophages after PAMP exposure

To identify potential ligands for CLAN, a number of PAMPs were used to assess the inflammatory response of the genetically manipulated THP-1 cell lines. The concentrations of PAMPs used were within the range typically required for bioactivity, as previously reported (14). In comparison with the control cell line THP-1/Neo, differentiated THP-1/CLAN cells secreted significantly higher amounts of processed IL-1β after exposure to LPS derived from *E. coli* and to PGN isolated from *S. aureus* (Fig. 2*A*). To exclude nonspecific suppression of PAMP response caused by introduction of the hairpin vector, a control cell line expressing a nonspecific (scrambled sequence) RNAi hairpin construct (THP-1/SCR) was also used in these experiments. After exposure to LPS or PGN, IL-1β secretion was not inhibited compared with that from THP-1/Neo control cells. In fact, the levels of IL-1β secreted from this cell line in response to PAMPs were significantly higher than those from THP-1/Neo cells. Addition of exogenous IFN-β to cultures before PAMP exposure also augmented PAMP responses by THP-1 cells (data not shown). Thus, nonspecific induction of IFN production by RNA hairpin vectors cannot account for the robust suppression of PAMP responses observed in THP-1 cells expressing the CLAN siRNA vector.

Dose-response analysis indicated that CLAN enhances IL-1β secretion after exposure to as little as 1 ng/ml LPS and 1 μg/ml PGN (Fig. 2, *B* and *C*). CLAN-overexpressing cells also secreted slightly more IL-1β after LTA exposure compared with control cells. In contrast to LPS, PGN, and LTA, another PAMP not typically associated with the induction of IL-1β secretion in human macrophages, unmethylated CpG DNA (26), did not stimulate the release of this cytokine from any of the cell lines examined.

Although the overexpression of CLAN enhanced THP-1 cell secretion of IL-1β in response to certain PAMPs, CLAN RNAi-expressing THP-1/HP cells exhibited a marked defect in IL-1β generation after exposure to LPS and PGN at all concentrations tested (Fig. 2, *A–C*). These findings suggest that endogenous CLAN plays a critical role in IL-1β production by THP-1 cells when confronted with bacterial cell wall components.

To explore the mechanism by which overexpression of CLAN leads to enhanced IL-1β secretion, we tested the effects of caspase inhibitory, cell-permeable peptides. In both THP-1/Neo and THP-1/CLAN cells, PAMP-induced IL-1β secretion was found to be dependent on caspase-1 activation, as shown by the ability of an irreversible inhibitor of this protease (z-WEHD-fmk) to abolish cytokine secretion (Fig. 2*D*). Preincubation with the pan-caspase inhibitor z-VAD-fmk also completely blocked IL-1β release after LPS and PGN exposure (data not shown). As measured by LDH release into the supernatant, no cytotoxicity was observed in treated cells during the course of these experiments (data not shown), suggesting that PAMP exposure selectively activates proinflammatory caspases, but not apoptotic caspases (reviewed in Ref. 27).

CLAN-dependent IL-1β secretion in THP-1 macrophages after bacterial infection

We next investigated whether modifying CLAN expression levels alters IL-1β secretion after bacterial infection. THP-1/Neo, THP-1/CLAN, and THP-1/HP cells were differentiated for 16 h using PMA, then infected with pathogenic Gram-negative bacteria in

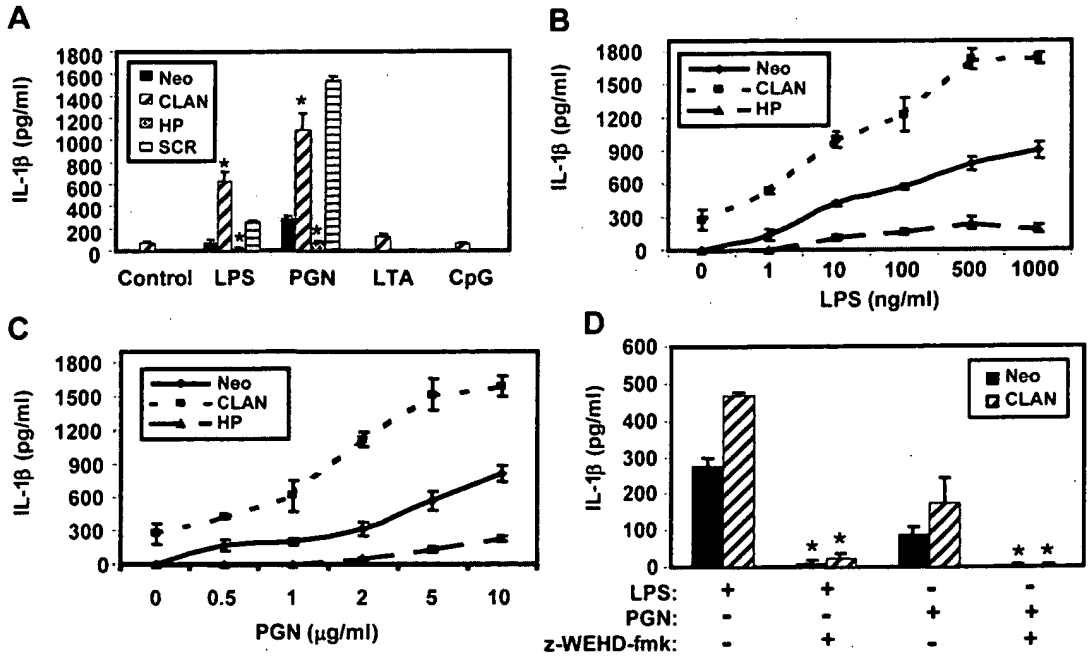

FIGURE 2. Effects of CLAN on IL-1β secretion in monocytic cell lines. *A*, Differentiated THP-1 cells (3×10^5) in 0.3 ml of medium were exposed to LPS (500 ng/ml), PGN (10 μg/ml), LTA (10 μg/ml), unmethylated CpG oligonucleotides (10 μM), or medium alone for 6 h at 37°C. Supernatants were collected and analyzed for bioactive IL-1β by ELISA, normalizing relative to cell number, as determined by the total protein content for each sample. ■, Neo; ▨, CLAN; ▩, RNAi; ▤, scrambled RNAi control. THP-1 cells were differentiated and exposed to varying concentrations of LPS (*B*) or PGN (*C*) for 6 h; supernatants were then collected and analyzed for active IL-1β by ELISA. Solid lines, Neo; dotted lines, CLAN; dashed lines, RNAi. *D*, Cells were pretreated for 2 h with z-WEHD-fmk (10 μM) or DMSO vehicle control before exposure to LPS or PGN. Supernatants were analyzed for IL-1β 6 h post-treatment. *, Statistically significant results ($p < 0.01$). Data shown represent the mean ± SD and are representative of three independent experiments. ■, Neo; ▨, CLAN.

log-phase growth at an MOI of 5. At 1 and 9 h after bacterial addition, cell supernatants were collected, and mature IL-1β levels were analyzed using ELISA.

After infection with *S. enteritidis* (LK5 strain), macrophages overexpressing CLAN secreted significantly higher amounts of IL-1β compared with control cells (Fig. 3*A*). In contrast to the enhanced response of THP-1/CLAN cells, THP-1/HP macrophages expressing CLAN RNAi consistently secreted slightly less IL-1β after *Salmonella* infection, although the results did not reach statistical significance. Similar results were obtained in experiments in which differentiated THP-1 cells were infected with *S. typhimurium* or *S. flexneri*, an invasive pathogen that (unlike *Salmonella*) does not reside in intracellular vacuoles (Fig. 3, *B* and *C*). *Salmonella*-induced IL-1β processing and secretion were rapid, occurring within 1 h of infection, most likely due to the efficient intracellular delivery of bacterial components and activation of a post-translational mechanism of cytokine regulation rather than to differences based on altered pro-IL-1β message levels. Elevated IL-1β release from THP-1/CLAN cells was observed at 9 h postinfection as well (Fig. 3, *A–C*).

Bacteria-induced secretion of IL-1β from THP-1/Neo cells was completely blocked by preincubating THP-1 cells with the caspase-1 inhibitor z-WEHD-fmk or the broad-spectrum caspase inhibitor z-VAD-fmk (Fig. 3*D*). Preincubation of THP-1/CLAN with z-WEHD-fmk reduced most, but not all, bacteria-induced IL-1β secretion, whereas z-VAD-fmk completely blocked IL-1β secretion for THP-1/CLAN in these experiments. These results suggest that after exposure to live bacteria, both caspase-1-dependent and -independent mechanisms of pro-IL-1β activation function in CLAN-overexpressing macrophages. Alternatively, these data may simply reflect differences in effectiveness of these pharmacological caspase inhibitors under the experimental conditions used.

Antibacterial effects of CLAN

To determine the effects of CLAN on intracellular bacteria levels within macrophages after infection, a gentamicin protection assay was used. THP-1 cells were differentiated for 16 h, then exposed to *Salmonella* species at an MOI of 5. After the killing of extracellular bacteria with gentamicin, cells were incubated for additional periods, then lysed to assess the number of viable intracellular bacteria capable of forming colonies when plated on Luria Bertoni/agar.

Overexpression of CLAN in THP-1 macrophages was associated with significantly reduced levels of surviving intracellular *S. enteritidis* compared with that in THP-1/Neo control cells (Fig. 4*A*). Similar observations were made using another strain of pathogenic bacteria, *S. typhimurium* (Fig. 4*B*), and a less virulent *S. enteritidis* strain (data not shown). Although THP-1/HP macrophages expressing CLAN RNAi often contained higher levels of intracellular bacteria in these experiments, the results did not reach statistical significance (not shown).

To further investigate the mechanism of the antibacterial effects of CLAN, THP-1 macrophages were pretreated with caspase inhibitors for 1 h before infection with *S. enteritidis*. Although the preadministration of z-WEHD-fmk or z-VAD-fmk lowered the overall levels of intracellular bacteria, no significant inhibition of the antibacterial effects of CLAN was observed, indicating a caspase-independent process. We also observed that the CFUs recovered from THP-1/Neo and THP-1/CLAN were approximately equivalent immediately after the infection (1 h), indicating that CLAN most likely does not significantly affect bacterial entry into

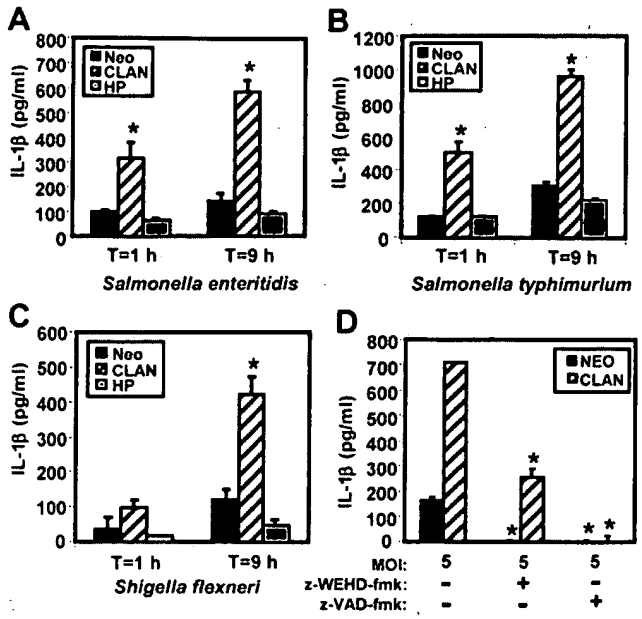

FIGURE 3. CLAN enhances IL-1β secretion after infection by pathogenic bacteria. THP-1 cells were differentiated by treatment with 50 ng/ml PMA for 16 h, then washed and infected with *S. enteritidis* strain LK5 (*A*), *S. typhimurium* (*B*), or *S. flexneri* (*C*) for 1 or 9 h at MOIs of 5, 5, and 10, respectively. Supernatants were collected and analyzed for IL-1β by ELISA. *D*, Cells were pretreated with z-WEHD-fmk (10 μM), z-VAD-fmk (50 μM), or DMSO vehicle control for 2 h before infection with *S. enteritidis* (MOI of 5) for 1 h (*, $p < 0.01$). Data shown represent the mean ± SD and are representative of three independent experiments. ■, Neo; ▨, CLAN.

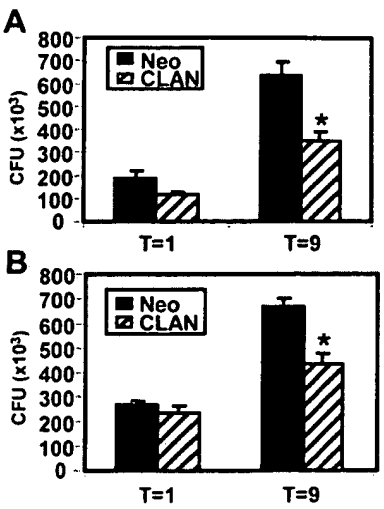

FIGURE 4. Antibacterial effects of CLAN in THP-1 macrophages. THP-1 cells were differentiated and infected with *S. enteritidis* (*A*) or *S. typhimurium* (*B*), both at an MOI of 5 for 1 h (T = 1). Extracellular bacteria were killed with gentamicin, and THP-1 cells were incubated for an additional 8 h (T = 9), after which surviving intracellular bacteria were recovered from THP-1 lysates and cultured overnight on Luria Bertoni-agar plates to determine the numbers of CFUs (*, $p < 0.01$). Data shown represent the mean ± SD and are representative of three independent experiments. ■, Neo; ▨, CLAN.

these cells (Figs. 4 and 5). To confirm this observation, THP-1 macrophages were grown on glass slides and infected with ECFP-expressing *S. typhimurium* for 1 h before extensive washing and microscopic determination of the percentage of macrophages with ingested bacteria. These studies indicated that the levels of initial phagocytosis of *Salmonella* by THP-1/Neo and THP-1/CLAN cells were approximately equal, thus ruling out altered bacterial uptake as a mechanism for the antibacterial phenotype of CLAN-overexpressing cells.

CLAN potentiates Salmonella*-induced cell death*

In pilot experiments involving *Salmonella* infection, we noted that THP-1/CLAN cells were rapidly killed after exposure to very high levels of bacteria (MOI ≥10). Cytotoxicity assays revealed that CLAN overexpression increased macrophage susceptibility to cell death induced by *S. enteritidis* and *S. typhimurium* at an MOI of 50, as determined by cytosolic LDH release into culture supernatants (Fig. 6, *A* and *B*). Infections of THP-1 cell lines with *Salmonella* species at an MOI of 10 produced similar results (data not shown). In contrast to live bacteria, heat-killed *S. typhimurium* applied at MOIs as high as 200 failed to induce any amount of cytotoxicity in THP-1/CLAN macrophages (data not shown).

Preincubation of THP-1 cell lines with z-WEHD-fmk failed to inhibit cell death after infection at a high MOI, suggesting a caspase-1-independent mechanism of cell death. In contrast, preincubation of THP-1/CLAN with the broad-spectrum caspase inhibitor z-VAD-fmk completely abolished cell death induced by *S. enteritidis* at a high MOI while greatly reducing cell death induced by high MOI *S. typhimurium* infection (Fig. 6, *A* and *B*). Despite the possibility of a caspase-1-independent cell death mechanism, an isogenic mutant of *S. typhimurium* lacking the caspase-1-activating SipB gene was also used and failed to effectively induce the death of any of the THP-1 cell lines tested (Fig. 6*C*).

Discussion

In this study we show that overexpression of CLAN in THP-1 macrophages enhances caspase-1-dependent IL-1β secretion induced by exposure to the bacterial cell wall components LPS and PGN, whereas ablation of CLAN expression using RNAi impairs IL-1β production by cells exposed to these PAMPs. Infection of CLAN-overexpressing macrophages with *S. enteritidis* or *S. typhimurium* also results in hypersecretion of IL-1β compared with that by control cells, demonstrating the ability of CLAN to modulate cytokine responses to live invading bacteria. When exposed at a modest MOI, CLAN overexpression provides a bacteriocidal or bacteriostatic effect, reducing the accumulation of live bacteria in infected macrophages. In contrast, when the intracellular bacterial burden is high, CLAN promotes macrophage cell death. Taken together, these results indicate that CLAN contributes to several biological processes that are central to host defense: modulating the relative sensitivity of macrophages to LPS and PGN, and impacting host-pathogen interactions.

During revision of this manuscript, Mariathasan et al. (28) demonstrated that macrophages from CLAN-deficient mice are defective in IL-1β secretion after infection by *S. typhimurium*. Thus, our data, using human cells and RNAi techniques, corroborate these findings from mice, establishing evolutionary conservation of mechanism. However, in contrast to results obtained using murine cells, where macrophages isolated from CLAN-deficient mice were found to secrete similar levels of IL-1β in response to purified PAMP molecules such as LPS and PGN (28), we found that genetic manipulation of CLAN expression in human cells did alter IL-1β production in response to individual PAMPs. This discrepancy may be due to the interspecies variation and warrants further investigation. Nevertheless, our findings suggest that endogenous CLAN plays a critical role in IL-1β production by human THP-1 cells when confronted with bacterial cell wall components.

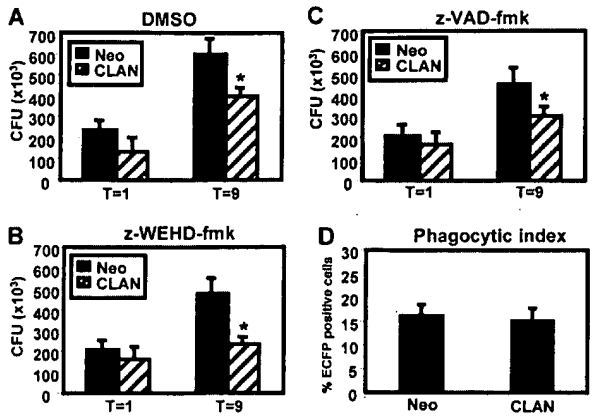

FIGURE 5. CLAN-induced antibacterial effects are independent of caspase activation or phagocytosis. THP-1 cells were differentiated and infected with *S. enteritidis* at an MOI of 5 after pretreatment with DMSO (*A*), z-WEHD-fmk (10 μM; *B*), or z-VAD-fmk (50 μM; *C*). After 1 or 9 h, CFU were determined (mean ± SD; $n = 3$). *D*, THP-1 cells were infected with ECFP-expressing *S. typhimurium* for 1 h for determination of the phagocytic index by fluorescence microscopy. Data shown represent the percentage of cells with ingested bacteria (mean ± SD) and are representative of two independent experiments performed in triplicate. ■, Neo; ▨, CLAN.

The activation of caspase-1 and its subsequent processing of proinflammatory cytokines represent critical events in the inflammatory process (13, 29) and may be associated with the pathogenesis of a variety of inflammatory diseases (30). In mammals, multiple proteins that control capase-1 activation have been identified (17, 31, 32). Among the caspase-1-activating proteins are intracellular proteins that respond to bacterial products, suggesting a role in innate immunity. CLAN, a macrophage-expressed protein with domain architecture similar to that of Nod1 and Nod2, is known to bind and activate caspase-1 in overexpression systems, but until now its functions have not been put into a physiological context.

The expression of endogenous CLAN is up-regulated in macrophages following exposure to the proinflammatory cytokine TNF-α, similar to observations made previously with regard to Nod2 expression in intestinal epithelial cells (33). Additionally, expression levels of CLAN were found to be up-regulated by the bacterial cell wall component LPS. The ability of TNF-α and LPS to increase CLAN expression indicates that it may be acutely increased in macrophages in preparation for or in response to bacterial challenge.

To explore the functions of CLAN in macrophages, we used gene transfer and RNAi-mediated gene ablation methods to examine the effects of manipulating CLAN expression in THP-1 macrophages. Our data indicate that the levels of CLAN determine the magnitude of THP-1 cell responses to several bacterial cell wall components (LPS, PGN, and LTA) with respect to release of IL-1β. The overexpression of CLAN also markedly enhanced the secretion of this cytokine from THP-1 macrophages after infection by live bacteria. Furthermore, these effects were suppressed by the caspase inhibitor z-WEHD-fmk, implying a caspase-1-dependent mechanism. Reducing CLAN expression by RNAi significantly diminished IL-1β production in response to bacterial cell wall components, but did not abolish the cellular response to live bacteria. We interpret these results to mean that either residual CLAN expression in RNAi-expressing cells was sufficient for retention of IL-1β production, or redundancy exists in the intracellular molecules that are capable of sensing the presence of bacteria and triggering caspase-1 activation and IL-1β production.

The observation that CLAN-overexpressing macrophages are hyper-responsive to LPS, PGN, and LTA suggests either that the LRR domain of CLAN recognizes structural elements common to all these molecules, or this NACHT family protein operates downstream of intracellular signaling pathways activated by all three PAMPs. Despite the large size of the NACHT protein family (with 20 members identified in the human genome) (34), to date only bacterial ligands have been identified for NACHT family members Nod1 and Nod2. The ability of CLAN to enhance IL-1β release in response to LPS, PGN, and LTA is significant in that these microbial components are believed to synergize in generating the inflammatory responses associated with septic toxic shock (1, 35). Thus, NACHT family members such as CLAN should be examined with respect to their potential to serve as targets for drug discovery, in particular, taking advantage of the putative nucleotide-binding NACHT domains, which are thought to mediate activation of these proteins via NACHT-NACHT oligomerization (15, 36, 37).

By monitoring levels of viable *Salmonella* in THP-1 macrophages after infection with these invasive bacteria, a role was discovered for CLAN in suppressing their intracellular growth or survival. Gentamicin protection assays using three different *Salmonella* species conclusively demonstrated that THP-1 cells

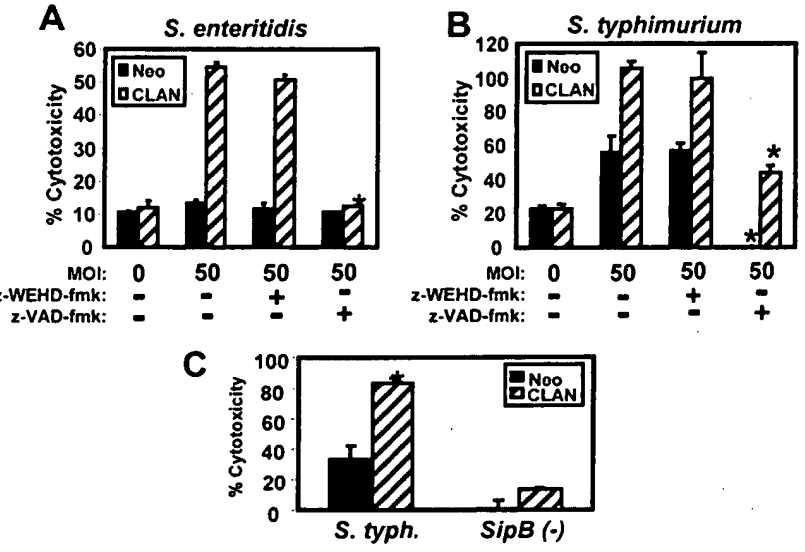

FIGURE 6. CLAN potentiates *Salmonella*-induced, caspase-dependent cell death in THP-1 macrophages. Differentiated THP-1 cells were pretreated with z-WEHD-fmk (10 μM), z-VAD-fmk (50 μM), or DMSO vehicle control for 2 h before infection with *S. enteritidis* (*A*) or *S. typhimurium* (*B*) for 2 h. Cytotoxicity was measured by cellular release of LDH into supernatants. Data are expressed as a percentage relative to total cellular LDH. *C*, Differentiated THP-1 cells were infected with *S. typhimurium* or a *S. typhimurium* SipB-deficient mutant (both at an MOI of 50). Cytotoxicity levels were assessed by LDH release (*, $p < 0.01$). Data shown represent the mean ± SD and are representative of three independent experiments. ■, Neo; ▨, CLAN.

overexpressing CLAN display enhanced antibacterial properties compared with control cells. No difference in *Salmonella* invasion was observed between THP-1/CLAN and THP-1/Neo cells. These results show that CLAN impacts processes that contribute to bacterial eradication or intracellular bacterial replication and are similar to the effects of Nod2 overexpression on *S. typhimurium* survival, as previously reported for intestinal epithelial cells (38). It remains to be determined whether various NACHT family proteins are responsible for recognizing individual pathogens and triggering subsequent antimicrobial activities or if they possess overlapping specificities. In an attempt to explain the inhibitory effects of CLAN on intracellular bacterial survival or proliferation, the levels of several endogenous antibacterial factors were analyzed in the THP-1 model. Specifically, the levels of human β-defensin-1 and -2 (natural antibiotic peptides previously shown to be regulated by LPS in macrophages) (39) were assessed in THP-1/Neo, THP-1/CLAN, and THP-1/HP cells by RT-PCR analysis. In untreated conditions and after exposure to *Salmonella* or LPS for various time periods, the expression levels of these genes were unchanged (not shown), thus excluding them as a possible explanation for the antimicrobial effects of CLAN.

Invasive pathogenic bacteria can find shelter from the host immune system by invading and replicating in macrophages, where they are protected from effectors of the humoral and innate immune defense systems. Bacteria such as *Salmonella* produce several virulence proteins associated with type III secretion systems that enable these microorganisms to subvert host antibacterial processes in the cytosol of phagocytes (40, 41). Another hypothesized benefit of residing within the macrophage is that these host cells carry organisms through the lymph and blood to other tissues, thus facilitating their in vivo dissemination (42, 43). Our observation that the overexpression of CLAN predisposes macrophages to cell death upon exposure to large bacterial loads suggests that this protein may serve to counter the "hitch-hiking" effect exploited by intracellular pathogens, similar to the hypersensitive response of plants (9). Conversely, it has been hypothesized that microbes such as *Salmonella* may trigger the apoptosis of host macrophages to induce tissue damage and facilitate pathogen spreading within the lymphatic system (44, 45). The fact that cell death, in addition to inflammation, is believed to be another dominant feature of septic shock lends further credence to the idea of targeting CLAN as part of a novel anti-inflammatory treatment (46).

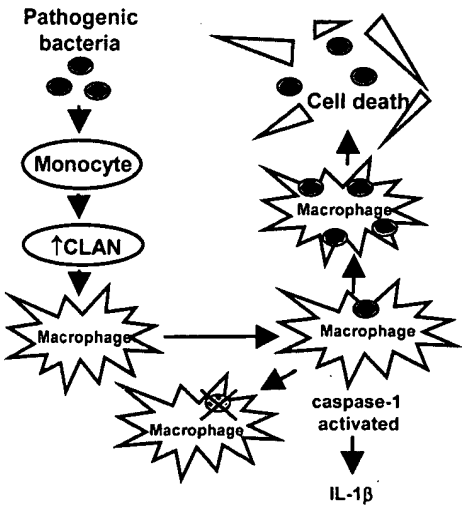

FIGURE 7. Model for CLAN function in human macrophages. Exposure of monocytes to bacteria (or bacterial products such as LPS) increases the expression of CLAN and induces differentiation to macrophages. Entry of bacteria into macrophages triggers caspase-1 activation and subsequent IL-1β secretion at least partially through CLAN. When the intracellular burden of bacteria is limited, CLAN potentiates bacteriocidal activity of macrophages (denoted by X). In contrast, when the bacterial load is high, CLAN promotes macrophage cell death.

The cytotoxicity experiments presented in this study demonstrate that *Salmonella*-induced cell death in CLAN-overexpressing human macrophages is rapid, with the majority of death occurring within the first hour of infection. The failure of CLAN RNAi expression in THP-1 cells to repress cell death induced by *Salmonella* raises the possibility that residual amounts of CLAN expression in siRNA-expressing cells may be sufficient to detect intracellular bacteria and induce cell death. Definitive evidence supporting a role for CLAN in inducing macrophage cell death was obtained recently by others using CLAN-deficient murine macrophages, showing failure to undergo cell death after *Salmonella* infection (28). Interestingly, the enhanced bacteria-induced cytotoxicity observed in CLAN-overexpressing THP-1 cells was refractory to suppression by the caspase-1 inhibitor, z-WEHD-fmk, implying a caspase-1-independent mechanism. Nevertheless, *Salmonella*-mediated killing of CLAN-overexpressing THP-1 macrophages is caspase-dependent, as demonstrated by its complete negation by the pan-caspase inhibitor z-VAD-fmk. In this regard, it may be relevant that CLAN has previously been reported to promote apoptosis by binding the bipartite adapter protein ASC (apoptosis-associated speck-like protein containing a CARD) and enhancing activation of procaspase-8 (47). Thus, CLAN may be capable of indirectly activating other members of the caspase family, besides caspase-1, accounting for our experimental observations. Interestingly, a role for caspase-2 in *S. typhimurium*-induced cell death has been suggested, with activation of this protease occurring in conjunction with caspases-3, -6, and -8 in murine bone marrow-derived macrophages (48). Given that exposure to high concentrations of LPS or heat-killed *Salmonella* did not induce cell death in CLAN-overexpressing macrophages, it seems that highly efficient intracellular presentation of PAMPs and/or a heat-labile factor are needed for cytotoxicity. The failure of a SipB-defective *Salmonella* strain to induce macrophage cell death may also indicate that a functional SipB-related virulence protein is required as a cofactor for *Salmonella*-induced killing of CLAN-overexpressing macrophages.

The influence of CLAN on multiple arms of the innate immune system indicates that this NACHT family protein may have diverse effects on invading pathogens at both the cellular level (through its antibacterial and host cell death-inducing effects) as well as at the level of the whole organism through proinflammatory cytokine secretion (summarized in Fig. 7). More detailed structural studies may elucidate the specific components of LPS and PGN to which CLAN-overexpressing cells respond, analogous to recent studies involving Nod1 and Nod2 in which the minimal elements of bacterial PGN were dissected (21, 24). The further characterization of genetically engineered mice lacking CLAN will help to conclusively define the role of CLAN in host defense against invading bacteria as well as in sepsis and inflammatory disease models.

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